

REMARKS

This amendment is in response to the Office Action, dated October 5, 2006 ("Office Action"). Following entry of the present amendment, claims 1, 110-118, 121-173, 176-200, and 204-221 are pending; claims 1, 118, 165, 168, 173, 184, 200, 204 and 205 having been amended; claims 119-120 and 174-175 having been canceled; and new claims 206-221 having been added by virtue of the present amendment (claims 1, 110-118, 121-173, 176-183, 188-190, and 192-199 were previously withdrawn; and claims 2-109 and 201-203 were previously canceled). No new matter has been added. Allowance and reconsideration of the application in view of Applicants' amendment and the ensuing remarks are respectfully requested.

Claims 1 and 165 have been amended such that they include all the limitations of claim 205.

Claims 118, 168, and 173 have been amended to recite that the agonist is one other than a metalloporphyrin.

Claim 184 has been amended to include YC-1 as one of the possible agonists of the calcium-activated potassium channel. Support may be found throughout the specification; for example, page 10, lines 16-20.

Claim 200 has been amended to recite that "an administration of the agonist and the medicant [provided by the kit] increases the permeability of a capillary or arteriole delivering blood to cells of the abnormal brain region to the medicant." Support may be found throughout the specification; for example, pages 7-9 and page 15, to name a few. The claim has also been amended to further recite that the agonist of a calcium-activated potassium channel may also be YC-1. Support may be found throughout the specification; for example, page 10, lines 16-20.

Claim 204 has been amended to recite that the agonist of a calcium-activated potassium channel is "other than a bradykinin, a bradykinin analog..." Support for this amendment may be found throughout the specification; for example, page 7, lines 10-14, page 8, lines 11-14, page 9, lines 18-22, page 14, lines 25-29 and page 15, lines 10-12.

Claim 205 has been amended to recite that “an administration of the agonist and the medicant [provided by the kit] increases the permeability of a capillary or arteriole delivering blood to cells of the abnormal brain region to the medicant.” Support may be found throughout the specification; for example, pages 7-9 and page 15, to name a few.

New composition claims 206-209 recite that the agonist of a calcium activated potassium channel is NS-1619, 1-EBIO, a guanylyl cyclase activating protein and YC-1, respectively.

New kit claims 210-213 similarly recite that the agonist of a calcium activated potassium channel is NS-1619, 1-EBIO, a guanylyl cyclase activating protein and YC-1, respectively.

New kit claim 214 recites that the kit comprises a composition and instructions, wherein the composition in the kit comprises an agonist of a calcium-activated potassium channel and a medicant; and also lists the agonists that may be in the kit. Support may be found throughout the specification; for example, page 8, lines 11-16, page 10, lines 14-21, page 14, lines 25-29, and page 15, lines 9-23.

New kit claims 215-218 recite that the agonist of a calcium activated potassium channel is NS-1619, 1-EBIO, a guanylyl cyclase activating protein and YC-1, respectively.

New kit claim 219 recites that the kit comprises a composition and instructions, wherein the composition in the kit comprises an agonist of a calcium-activated potassium channel, other than a bradykinin, a bradykinin analog or a metalloporphyrin, and a medicant. Support may be found throughout the specification; for example, page 7, lines 10-14, page 8, lines 11-16, , page 9, lines 18-22, page 10, lines 14-21, page 14, lines 25-29, and page 15, lines 9-23.

New dependent method claims 220 and 221 recite that “the agonist is formulated together in a pharmaceutically acceptable solution with the medicant and the medicant is a drug for delivery by intravascular infusion or injection...and wherein administering to the mammalian subject comprises administering the agonist that is formulated together in a pharmaceutically acceptable solution with the medicant such that the agonist is administered simultaneously with the medicant. The claims also recite a list of drugs that may be used. Thus, these claims include the limitations of claim 204.

Examiner rejected claims 184, 185, 187, 191 and 200 under 35 U.S.C. §112, first paragraph for alleged non-enablement for the use of a guanylyl cyclase activating protein as a calcium-activated potassium channel agonist. Examiner found that “it is conceivable that activators of guanylate cyclase could indirectly act as K_{Ca} channel agonists by increasing intracellular cGMP and subsequently activating cGMP-PK.” However, Examiner, citing Ermilov, asserted that “GCAPs interact with the intracellular domain of guanylyl cyclase.” Examiner further cited Hwang *et al.* and Frins *et al.* and asserted that “extracellularly administered GCAPs would [not] activate cellular guanylate cyclase because the intracellular guanylate cyclase domains with which GCAPs naturally interact would not be accessible to extracellularly administered GCAPs.” Examiner further stated that “because GCAPs act intracellularly to activate guanylyl cyclases, one of skill in the art could not use them for their intended purpose of activating K_{Ca} channels absent some guidance as to how to cause them to be internalized into a cell,” and thus would require undue experimentation. Applicants respectfully traverse this rejection.

Applicants respectfully disagree with Examiner’s assertion and respectfully submit the specification indeed teaches one of skill in the art to use a GCAP and a K_{Ca} channel agonist without undue experimentation. One of skill in the art would indeed recognize without undue experimentation that extracellularly administered GCAPs can activate cellular guanylate cyclase. For example, extracellular circulation of atriopeptin II (*i.e.*, a GCAP), an atrial natriuretic peptide fragment, has been found to activate guanylyl cyclase. See Currie *et al.*, “Purification and sequence analysis of bioactive atrial peptides (atriopeptins),” *Science* 1984 Jan 6; 223(4631):67-9; see also Sigma-Aldrich’s product description for Atriopeptin II (www.sigmaaldrich.com/catalog/search/ProductDetail/SIGMA/A9035) (collectively as Exhibit A). Applicants also respectfully submit that one of skill in the art will find that guanylyl cyclase activating proteins may in fact cross the plasma membrane and start the cascade of reactions to activate the calcium-dependent potassium channels. For example, GCAPs may be soluble fusion proteins that are known in the art. See Sokal *et al.*, “Soluble Fusion Proteins between Single Transmembrane Photoreceptor Guanylyl

Cyclases and Their Activators," *Biochemistry* 2002, 41(1):251-257 (exhibit B). The soluble fusion proteins may serve to activate guanylyl cylase which indirectly activates the calcium-activated potassium channels. Additionally, cells frequently possess mechanisms for the uptake of proteins and one of skill in the art would appreciate that extracellularly administered guanylyl cyclase activating protein may cross the plasma membrane to activate guanylyl cyclase. Furthermore, a GCAP may interact with the extracellular domain of guanylyl cyclase. See Rambotti *et al.*, "Ultracytochemistry as a tool for the study of the cellular and subcellular localization of membrane-bound guanylate cyclase (GC) activity. Applicability to both receptor-activated and receptor-independent GC activity." *Molecular and Cellular Biochemistry* 2002, 230(1-2):85-96 (exhibit C). As such, Applicants respectfully request reconsideration and withdrawal of this rejection.

Examiner rejected claims 200 and 205 under 35 U.S.C. §103(a) as being unpatentable over Veltkamp *et al.* (Stroke 29: 837-843, 1998) and over Devor *et al.* (Am. J. Physiol. 271(5): L775-84, 1996). Examiner found that Veltkamp *et al.* taught "methods for assaying the effects of NS-1619 on the vascular response to NMDA after hypoxia and ischemia." Examiner found that Devor *et al.* "evaluated the effects of 1-EBIO and charybdotoxin on chloride ion secretion in T82 monolayers." Examiner found that although Veltkamp *et al.* and Devor *et al.* did not teach organizing NS-1619 and NMDA, or 1-EBIO and charybdotoxin into a kit, it would have been obvious to one skilled in the art to organize the agents because one skilled in the art would appreciate that "organizing experimental reagents prior to use is standard laboratory practice which reduces the frequency of errors." Examiner also noted that "instructions for use" did not impart patentable weight because application of printed matter to an old article cannot render the article patentable. Examiner was not persuaded by Applicants' previous arguments against this rejection. Examiner stated that there has been no evidence or reason to indicate that NMDA is not a medicant. Examiner further found that charybdotoxin, even as an inhibitor of 1-EBIO that would defeat the purpose of administering 1-EBIO to the abnormal brain region, is "not determinative of whether it would be obvious to include these reagents in a kit." Examiner stated that the relevant

question is “whether or not it would have been obvious to organize the components taught by Devor into a kit.” Applicants respectfully traverse this rejection.

Three criteria must be met to establish a *prima facie* case of obviousness: (1) “there must be some suggestion or motivation . . . to combine reference teachings,” (2) “there must be a reasonable expectation of success,” and (3) the prior art references “must teach or suggest all the claim limitations.” MPEP § 2142 (emphasis added). Moreover, “[t]he teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant’s disclosure.” *Id.* (citing *In re Vaeck*, 947 F.2d 488 (Fed. Cir. 1991); emphasis added).

Applicants respectfully submit that Veltkamp *et al.* does not render it obvious to organize an agonist of the calcium-activated potassium channel, a medicant, and instructions to use the agonist, into a kit. Further, claims 200 and 205, as amended, recite that “an administration of the agonist and the medicant increases the permeability of a capillary or arteriole delivering blood to cells of the abnormal brain region to the medicant.” These claims do not merely state an intended use; these claims recite a result that flows from the use of the components of the kit. Veltkamp *et al.* does not teach that the use of NS-1-EBIO and charybdotoxin would result in an “increas[ing] the permeability of a capillary or arteriole delivering blood to cells of the abnormal brain region to the medicant.” Veltkamp *et al.* does not provide any motivation to organize an agonist of the calcium-activated potassium channel, a medicant, and instructions to use the agonist into a kit, whereby an administration of the agonist and the medicant increases the permeability of a capillary or arteriole delivering blood to cells of the abnormal brain region to the medicant. Moreover, Applicants reiterate that Veltkamp *et al.* does not teach the use of NMDA as a medicant and Applicants respectfully submit that NMDA may not be a medicant. A literature search did not yield any evidence of NMDA as a medicant. On the contrary, one of skill in the art will appreciate that NMDA may not be used as a medicant because it activates NMDA receptors and glutamate receptors to release glutamate and excessive glutamate release causes neurotoxicity. Furthermore, Veltkamp *et al.* does not teach or suggest all the limitations of claims 200 and 205.

Applicants respectfully submit that Devor *et al.* similarly does not render it obvious to organize an agonist of the calcium-activated potassium channel, a medicant, and instructions to use the agonist, into a kit. Claims 200 and 205, as amended, recite that the administration of the agonist and the medicant increases the permeability of a capillary or arteriole delivering blood to cells of the abnormal brain region. These claims do not merely state an intended use; these claims recite a result that flows from the use of the components of the kit. Devor *et al.* does not teach that the use of charybdotoxin and 1-EBIO would result in an “increas[ing] the permeability of a capillary or arteriole delivering blood to cells of the abnormal brain region to the medicant.” Devor *et al.* does not provide any motivation to organize an agonist of the calcium-activated potassium channel, a medicant, and instructions to use the agonist into a kit, whereby an administration of the agonist and the medicant increases the permeability of a capillary or arteriole delivering blood to cells of the abnormal brain region to the medicant. In fact, Devor *et al.* teaches away from the invention because it teaches that charybdotoxin is an inhibitor of 1-EBIO (see Devor *et al.*, page L775). Thus, one of skill in the art would not be motivated to organize charybdotoxin and 1-EBIO into a kit with an expectation that the administration of the agonist and the medicant increases the permeability of a capillary or arteriole delivering blood to cells of the abnormal brain region. Furthermore, Devor *et al.* does not teach or suggest all the limitations of claims 200 and 205.

In light of the foregoing, Applicants respectfully request reconsideration and withdrawal of this rejection as to amended claims 200 and 205.

Further, while Applicants in no way concede that it would have been obvious to organize NS-1619 and NMDA, or 1-EBIO and charybdotoxin into kits, Applicants respectfully submit that neither Veltkamp *et al.* nor Devor *et al.* render obvious new kit claims 214-219. These claims are directed to a kit that comprises a composition and instructions to use the composition, wherein the composition comprises an agonist of the calcium-activated potassium channel and a medicant. Veltkamp *et al.* used NS-1619 and NMDA separately in the experiment as one group of experimental animals were pretreated with NS1619 prior to administration of NMDA (see page 838). Similarly, Devor *et al.* used 1-EBIO and charybdotoxin separately in the experiment as

the cells were treated with 1-EBIO and then charybdotoxin was added to inhibit the response that was stimulated by 1-EBIO (see page L777). Thus, Veltkamp *et al.* and Devor *et al.* do not teach or suggest having an agonist of the calcium-activated potassium channel and a medicant as one composition. Accordingly, Veltkamp *et al.* and Devor *et al.* do not teach or suggest each and every claim limitation in claims 200 and 205.

Examiner further rejected claim 204, under 35 U.S.C. §103(a) as being unpatentable over one of Black (U.S. Pat. 5,524,778) or Black (U.S. Pat. 5,434,137). These patents disclose the use of bradykinin as an agonist of the potassium channel. Examiner found that while the patents “do not explicitly disclose a composition comprising the bradykinin and the neuropharmaceutical or diagnostic agent, [] it would have been obvious.” Applicants respectfully traverse this rejection.

Applicants have amended claim 204 to recite that the potassium channel agonist is one other than bradykinin or a bradykinin analog. As such, this rejection is rendered moot. Applicants respectfully request reconsideration and withdrawal of this rejection as to amended claim 204.

Examiner also issued a nonstatutory double patenting rejection for claim 204 as being unpatentable over U.S. Patent Nos. 5,434,137, U.S. Patent No. 5,527,778, 6,043,223. Applicants respectfully traverse this rejection.

Applicants have amended claim 204 to recite that the potassium channel agonist is one other than bradykinin or a bradykinin analog. As such, this rejection is rendered moot. Applicants respectfully request reconsideration and withdrawal of this rejection as to amended claim 204.

Applicants also respectfully draw Examiner’s attention to the following:

“Where product and process claims drawn to independent and distinct inventions are presented in the same application, applicant may be called upon under 35 U.S.C. § 121 to elect claims to either the product or process . . . However, if applicant elects claims directed to the product,

and a product claim is subsequently found allowable, withdrawn process claims which . . . include all the limitations of the allowable product claim will be rejoined." (emphasis added) (MPEP § 821.04)

Applicants' independent process claims 1 and 165 (Group I in Examiner's previous restriction requirement) include the limitations of claim 205 (falling into Group II in Examiner's previous restriction requirement). Applicants' dependent process claims 219 and 220 further include the limitations of product claim 204. Furthermore, Applicants respectfully submit that product claims 204 and 205 are allowable. Therefore, Applicants respectfully submit that the claims of Group I, drawn to a non-elected process for using the products described in claim 204 or 205, and including all of the limitations thereof, must be rejoined in the present application.

All of the claims remaining in the application are now believed to be allowable. Favorable consideration and a Notice of Allowance are earnestly solicited.

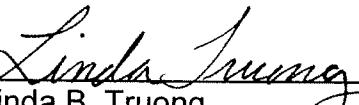
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If questions remain regarding this application, the Examiner is invited to contact the undersigned at (213) 633-6800.

Respectfully submitted,

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Attachments:

Exhibit A: Currie *et al.* "Purification and sequence analysis of bioactive atrial peptides (atriopeptins)," *Science* 1984; 223(4631):67-9, abstract only and Sigma-Aldrich's product description for atriopeptin II.

Exhibit B: Sokal *et al.*, "Soluble Fusion Proteins between Single Transmembrane Photoreceptor Guanylyl Cyclases and Their Activators," *Biochemistry* 2002, 41(1):251-257.

Exhibit C: Rambotti *et al.*, "Ultracytochemistry as a tool for the study of the cellular and subcellular localization of membrane-bound guanylate cyclase (GC) activity. Applicability to both receptor-activated and receptor-independent GC activity," *Molecular and Cellular Biochemistry* 2002, 230(1-2):85-96, abstract only.

Request for Continued Examination

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1: *Br J Pharmacol*. 1995 Apr;114(8):1587-94.

Vasorelaxant effect of isoliquiritigenin, a novel soluble guanylate cyclase activator, in rat aorta.

Yu SM, Kuo SC.

Department of Pharmacology, Chang Gung Medical College, Kwei-San, Tao-Yuan.

1. The vasorelaxant activity of isoliquiritigenin, isolated from *Dalbergia odorifera* T, was investigated in the phenylephrine-precontracted rat aorta by measuring tension, guanylate and adenylate cyclase activities, guanosine 3':5'-cyclic monophosphate (cyclic GMP) and adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels. 2. Isoliquiritigenin concentration-dependently relaxed rat aorta contracted with phenylephrine, KCl, U-46619, endothelin and 5-hydroxytryptamine, with EC50s of 7.4 +/- 1.6, 10.5 +/- 2.3, 14.3 +/- 3.3, 11.8 +/- 2.0 and 13.6 +/- 3.7 microM, respectively. 3. Isoliquiritigenin caused endothelium-independent relaxation of phenylephrine-precontracted rat aortic rings. Neither NG-monomethyl-L-arginine (L-NMMA) (an inhibitor of the L-arginine-NO pathway) nor oxyhaemoglobin (which binds NO) modified the relaxant effect of isoliquiritigenin. The relaxant action of isoliquiritigenin also persisted in intact aorta in the presence of indomethacin or glibenclamide. However, methylene blue, an inhibitor of soluble guanylate cyclase, abolished relaxation induced by isoliquiritigenin. 4. Incubation of rat aorta with isoliquiritigenin not only increased aortic cyclic GMP content but also caused small increases in aortic cyclic AMP content, and greatly potentiated the increases in cyclic AMP observed in the presence of forskolin. The maximum increase in cyclic GMP by isoliquiritigenin was reached earlier than the increase in cyclic AMP. This result suggests that the increases in cyclic GMP caused by isoliquiritigenin might stimulate the accumulation of cyclic AMP. 5. Concentration-dependent increases in soluble guanylate cyclase activity were observed in isoliquiritigenin (1-100 microM)- or sodium nitroprusside (SNP)-treated rat aortic smooth

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Endothelium-dependent relaxation of rat aorta by butein, a novel cyclic AMP specific phosphodiesterase inhibitor. [Eur J Pharmacol. 1995;285(2):211-6.]

Effects of cyclic GMP elevators on isoprenaline-induced increase in cyclic AMP and relaxation in rat aortic smooth muscle: role of phosphodiesterase 3. [Br J Pharmacol. 1995;114(8):1587-94.]

A xanthine-based KMUP- with cyclic GMP enhancer and K(+) channels open activities in rat aortic sm muscle. [Br J Pharmacol. 1995;114(8):1587-94.]

Involvement of cyclic nucleotide-dependent protein kinases in cyclic AMP-mediated vasodilation. [Br J Pharmacol. 1995;114(8):1587-94.]

Cardiovascular effects of novel, potent and selective phosphodiesterase 5 inhibitor DMPO: in vitro and in vivo characterization. [Br J Pharmacol. 1995;114(8):1587-94.]

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muscle cells, while adenylate cyclase activity was unchanged in isoliquiritigenin (100 microM)-treated cells.(ABSTRACT TRUNCATED AT 250 WORDS)

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Molecular Formula	C ₁₂₇ H ₂₀₃ N ₄₅ O ₃₉ S ₃
Molecular Weight	3080,44
CAS Number	91917-63-4
MDL number	MFCD00076226

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Reference	Gonzalez Bosc, L.V., Effects of atrial natriuretic peptide in the gut. <i>Peptides</i> 21 , 875, (2000)
Palluk, R., et al., Atrial natriuretic factor. <i>Life Sci.</i> 36 , 1415-1425, (1985)	

Potter, L.R., and Hunter, T., Identification and characterization of the phosphorylation sites of the guanylyl cyclase-linked natriuretic peptide receptors A and B. *Methods* **19**, 506-520, (1999)

Potter, L.R., Hunter, T., Structural and functional evolution of the natriuretic peptide system in vertebrates. *Int. Rev. Cytol.* **194**, 1, (2000)

Thibault, G., et al., Regulation of natriuretic peptide secretion by the heart. *Annu. Rev. Pharmacol. Toxicol.* **61**, 193, (1999)

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Soluble Fusion Proteins between Single Transmembrane Photoreceptor Guanylyl Cyclases and Their Activators[†]

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Abstract

Among single-spanning transmembrane receptors (sTMRs), two guanylyl cyclase receptors, GC1 and GC2, are critically important during phototransduction in vertebrate retinal photoreceptor cells. Ca^{2+} -free forms of guanylyl cyclase-activating proteins (GCAPs) stimulate GCs intracellularly by a molecular mechanism that is not fully understood. To gain further insight into the mechanism of activation and specificity among these proteins, for the first time, several soluble and active truncated GCs and fusion proteins between intracellular domains of GCs and full-length GCAPs were generated. The GC activity of myristoylated GCAP_{1–437–1054}GC displayed typical $[\text{Ca}^{2+}]$ dependence, and was further enhanced by ATP and inhibited by guanylyl cyclase inhibitor protein (GCIP). The myristoyl group of GCAP1 appeared to be critical for the inhibition of GCs at high $[\text{Ca}^{2+}]$, even without membranes. In contrast, calmodulin (CaM)_{1–437–1054}GC1 fusion protein was inactive, but could be stimulated by exogenous GCAP1. In a series of experiments, we showed that the activation of GCs by linked GCAPs involved intra- and intermolecular mechanisms. The catalytically productive GCAP1_{1–437–1054}GC1 complex can dissociate, allowing binding and stimulation of the GC1 fusion protein by free GCAP1. This suggests that the intramolecular interactions within the fusion protein have low affinity and are mimicking the native system. We present evidence that the mechanism of GC activation by GCAPs involves a dimeric form of GCs, involves direct interaction between GCs and GCAPs, and does not require membrane components. Thus, fusion proteins may provide an important advance for further structural studies of photoreceptor GCs and other sTMRs with and without different forms of regulatory proteins.

The intracellular regulation of single transmembrane receptors (sTMRs) by soluble proteins is at the heart of many fundamental cellular processes. For example, tyrosine autophosphorylation

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of growth hormone sTMRs and other related receptors modulate their own kinase activity. Consequently, autophosphorylated sTMRs recruit intracellular proteins to plasma membranes, including phospholipase C and ras GTPase-activating protein, or proteins containing Src homology domains (1). In vertebrate retina, sTMR guanylyl cyclases GC1 and GC2 are responsible for the production of the internal messenger cGMP, which is depleted via photoactivated rhodopsin/G-protein-mediated stimulation of phosphodiesterase. The increase production of cGMP occurs concomitantly with lower intracellular $[Ca^{2+}]$, which is detected by small Ca^{2+} -binding proteins (CBPs), termed guanylyl cyclase-activating proteins (GCAP1–3), that in turn regulate GCs (2–4). GCAP1 and GCAP3 are more closely related than GCAP2; however, these three proteins share strong similarity in overall topology. They are myristoylated and contain four EF-hand motifs with EF1 nonfunctional, as a consequence of the lack of key residues involved in Ca^{2+} coordination. GCAP1 and GCAP2 are rod- and cone-specific proteins (2–4), while GCAP3 is only expressed in cones (Imanishi et al., unpublished results).

Regulation of GCs could be considered as a model system for understanding the molecular mechanisms of intracellular interactions between sTMRs and intracellular CBPs. In addition, the malfunction of this regulation may lead to severe signal transduction abnormalities. For example, missense mutations constitutively activating GCAP1 or disabling mutations of GC1 lead to retina dystrophies and blindness (5–9).

Photoreceptor GCs are single-spanning plasma or internal membrane receptors and are thought to be associated with cytoskeletal structures of rod and cone outer segments (10, 11). For photoreceptor GCs, the intracellular regulation by GCAPs is so far the only known mechanism that controls enzymatic activity. In contrast, for other nonretinal GCs, the best-characterized mechanism of regulation of enzymatic activity is through binding of a peptide to the extracellular domain of GCs (12). All GCs display a significant relationship to vertebrate adenyl cyclases (ACs) with respect to the catalytic mechanisms of cyclic nucleotide synthesis, including a conserved set of key residues involved in enzymatic catalysis (13, 14), although the transmembrane segments of ACs and GCs are dissimilar (15–17). Various AC isozymes are also regulated in a subtype-specific manner by intracellular proteins, such as G-protein α - and $\beta\gamma$ -subunits, calmodulin (CaM) (18), and, interestingly, by a GCAP-like protein, termed VILIP1 (19, 20). Here, we use novel fusion proteins between photoreceptor GCs and CBPs to understand the mechanism and regulation of cGMP production. We found that activation by GCAPs involves a dimeric form of GC, direct interaction between GCs and GCAPs, and that the membrane components are dispensable during this process. This novel approach may provide an important step for further structural studies, not only for photoreceptor GCs with and without different forms of regulatory proteins, but also for other sTMRs.

MATERIALS AND METHODS

Cloning of the Intracellular Domain of Bovine GC1 and GC2. PCR products were cloned into pCRII-TOPO vector (TOPO TA Cloning Kit, Invitrogen) and sequenced by the dyedeoxyterminator method (ABI-Prism, Perkin-Elmer).

A *Kpn*I site was introduced at the N-terminus of the intracellular domain of bovine GC1 by PCR with primers GCa (5'-CGGTACCAGGCACCGGCTGCTTCACATCCA-3') and GCb (5'-CCCTCTTCACCACCTCCTCGGGA-3'). The intracellular domain of bovine GC1 (amino acids 437–1054) was then transferred between sites *Kpn*I and *Bam*HI of the pET30b vector (Novagen) in two fragments, *Kpn*I–*Xmn*I (PCR fragment) and *Xmn*I–*Bam*HI, covering the C-terminus of bovine GC1.

The intracellular domain of retGC2 (amino acids 437–1054) was amplified from a bovine retina cDNA library in two fragments with primers GC6 (5'-GGTACCAGACATCGATAAATAAAATCCAG-3') and GC9 (5'-CAAAGCTATAGACGTCTCCTG-3') and primers GC8 (5'-CAGGAGACGTCTATAGCTTTG-3') and GC10 (5'-GTCGACTCACCAGCTGCTTTCTGC-3'). These *Kpn*I–*Aat*II and *Aat*II–*Sal*I fragments covering the intracellular domain of retGC2 were then transferred between the *Kpn*I and *Sal*I sites of pET30b.

The ^{437–1054}GC1 and ^{437–1054}GC2 fused to a His₆-tag/thrombin/S-tag/enterokinase peptide linker (36 amino acids long, MHHHHHHSSGLVPRGSGMKGTAAGKFERQHMDSPDL) were transferred as a *Xba*I–*Xho*I fragment into the pFastBac1 expression vector (Bac-to-Bac system, Life Technologies, Inc.). SF9 insect cells were transfected with the recombinant bacmids using cationic liposome-mediated transfection (CellFECTIN reagent, Life Technologies, Inc.). Expression of recombinant proteins was tested 3 days after infection.

Cloning of the Truncated Intracellular Domain of GC1. Truncated GC1 was amplified from a bovine retina cDNA library into two fragments. ^{468–1054}GC1 was amplified by PCR with primers PL4 (5'-ATGGGGAATTCTCGAAAGGTGGCCAG-3') and PL7 (5'-GGATCAGATCTCCAGGTTACTG-3') and primers PL6 (5'-ATGGAAGATCTGATCCGGGAGCGCACA-3') and XE2 (5'-GAATTCTCACTCCCAGAAAC-3'). ^{514–1054}GC1 was cloned by PCR with primers GC19 (5'-GATATCCCAGGAGATCGGCATATAG-3') and PL7 and primers PL6 and XE2. These *Eco*RI–*Bg*II or *Eco*RV–*Bg*II fragments, for ^{468–1054}GC1 and ^{514–1054}GC1, respectively, were cloned with the *Bg*II–*Eco*RI fragment into pET30b-opened *Eco*RI or *Eco*RV–*Eco*RI.

Cloning of GCAP–GC Fusion Proteins. Bovine GCAP1, GCAP2, or CaM was fused to the intracellular domain of bovine GC1 or GC2 in the pET30b vector. The coding sequences of bovine GCAP1, GCAP2, and CaM were amplified by PCR to remove the stop codon using primers FH23 (5'-CATATGGGGAACATTATGGAC-3') and LN1 (5'-CATATGACCGTCGGCCTCCGCG-3') for GCAP1, primers GCAP2U (5'-CACGGATCCATGGGGCAGCAGTTCAGCTGGGAG-3') and LN2 (5'-CATATGGAACATGGCACTTT-3') for GCAP2, and primers FH30 (5'-CATATGGCTGACCAACTCAC-3') and KP142 (5'-CATATGCTTAGCCGTACATTTG-3') for CaM. The PCR was heated for 5 min at 94 °C, followed by 25 cycles at 94 °C for 30 s, primer specific temperatures for 30 s, and 68 °C for 2 min followed by 7 min at 68 °C. GCAP1 and CaM were then cloned into the *Nde*I site, GCAP2 between the *Xba*I and *Nde*I sites of pET30b vector upstream of the 36 amino acid long linker–^{437–1054}GC1, –^{468–1054}GC1, –^{514–1054}GC1, or –^{437–1054}GC2. The GCAP–linker–GC fusion proteins were then transferred as fragments *Xba*I–*Xho*I into the pFastBac1 vector and expressed in insect cells as described above.

Cloning of His-tag–, PPE-tag–, and Strep-tag–GCAP–GC Fusion Proteins. *Nco*I and *Nde*I restriction sites were inserted by PCR, respectively, on the ATG or before the stop codon of bovine GCAP1. Annealed oligonucleotides (5'-CTAGATTATGGCCCCCGTGGACCGGACCGACCCGAGGGATCGAGGAGGC-3' and 5'-CATGGCCTCCTCGATCCCCTCGGGTCCGGTCCACGCGGGGCCATAAT-3') encoding the “PRGPDRPEGIEE” HIV peptide fragment (PPE–GC1), that introduced *Xba*I and *Nco*I protruding ends, were cloned with the fragment *Nco*I–*Nde*I encoding GCAP1 between *Xba*I and *Nde*I in pET30b upstream of the 36 amino acid long linker–^{437–1054}GC1.

The His₆-tag was inserted at the GCAP1 5'-end by PCR with primers FH210 (5'-CATATGCACCATCACCATGGAACATTATGGACGGTAAG-3') and LN1 (5'-CATATGACCGTCGGCTCCGCG-3') that also introduced *Nde*I sites. The *Nde*I-*Nde*I GCAP1 fragment was then subcloned in pET30b upstream from the 36 amino acid long linker-⁴³⁷⁻¹⁰⁵⁴GC1.

Strep-tag, a short peptide (8 amino acids long) with highly selective binding properties for Strep-Tactin (an engineered streptavidin), was also fused to the N-terminus of GCAP1-linker-⁴³⁷⁻¹⁰⁵⁴GC1 (21). Annealed oligonucleotides (5'-GATCCAATATGGCTAGCAACTGGAGCCACCCGCAATTGAAAAAGGCGGGCA-3' and 5'-TATGCCCGCCTTTCAAATTGCGGGTGGCTCCAGTTGCTAGCCATATTG-3') encoding the “MASN-WSHPQFEKGG” Strep-tag peptide, that introduced *Bam*HI and *Nde*I protruding ends, were cloned with the *Nde*I-*Xba*I fragment encoding linker-⁴³⁷⁻¹⁰⁵⁴GC1 or GCAP1-linker-⁴³⁷⁻¹⁰⁵⁴GC1 in pFastBac-opened *Bam*HI-*Sal*I.

GC Activity Assay and Protein Purification. Washed rod outer segment (ROS) membranes (22) were prepared from fresh bovine retinas (Schenk Packing Co., Stanwood, WA) reconstituted with recombinant GCAPs and assayed as described previously (23). [Ca²⁺] was calculated using the computer program Chelator 1.00 (24). All assays were repeated at least twice.

All purification procedures were carried out at 5 °C or on ice. Recombinant GCs and GCAPs were expressed in SF9 insect cells. After homogenization, cell suspensions were centrifuged at 10000g for 20 min at 4 °C. Supernatants were used as a source of recombinant proteins for protein purification. GCAPs and GCAP-GC fusion proteins were purified by Ni²⁺-nitrilotriacetic acid (Ni²⁺-NTA) metal-affinity chromatography (Qiagen). The cells were harvested and homogenized with 10 mL (10 plates, 15 cm in diameter) of 10 mM BTP, pH 7.5, containing 2 mM benzamidine. After centrifugation for 10 min at 35000g, the supernatant was incubated for 90 min at 4 °C with 1 mL of Ni-NTA resin. The resin was washed with the same buffer until the absorption at 280 nm was <0.02, and then washed with 10 mM BTP, containing 300 mM NaCl, until A_{280} nm < 0.02 and eluted with 10 mM Hepes, pH 7.5, containing 100 mM NaCl and 250 mM imidazole. Purified proteins were dialyzed against 10 mM BTP, pH 7.5, containing 50 mM NaCl overnight at 4 °C. The purity of proteins (>90% for GCAPs and >60% for GCAPs-GCs) was estimated by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie staining.

PPE-GCAP1-⁴³⁷⁻¹⁰⁵⁴GC1 protein was purified using antibodies against anti-PPE peptide (PRGPDRPEGIEE) coupled to CNBr-activated Sepharose resin (~5 mg of IgG per 1 mL of the gel) as described previously (25). Insect cells were homogenized in 10 mM BTP, pH 7.5, and centrifuged (10 min, 10000g, 4 °C), and the supernatant from 10 plates (15 cm) of SF9 cell culture infected with baculovirus-containing GCAP-GC constructs was loaded onto an antibody-Sepharose column (0.8 mg of the gel) equilibrated with 10 mM BTP, pH 7.5, at a flow rate of 6 mL/h. The column was then washed with 10 mM BTP, pH 7.5, containing 200 mM NaCl, and then with 10 mM BTP, pH 7.5. The elution was performed with 1 mM PRGPDRPEGIEE peptide in 10 mM BTP, pH 7.5, containing 50 mM NaCl. Fractions (0.5 mL) were collected and analyzed for GC-stimulating activity and proteins visualized by SDS-PAGE and immunoblotting (Figure 3). Typically, 100 µg of GCAPs and 5–10 µg of GCAP-GC fusion protein were obtained from 10 plates of monolayer SF9 insect cell culture (15 cm in diameter). GCAP-GC proteins were stable for >12 h.

For the dilution experiments, the amount of cGMP was determined by standardized Mono Q column chromatography (Pharmacia) using unlabeled GTP as a substrate. Samples (50 μ L in 950 μ L of 10 mM BTP, pH 7.5) were loaded on the Mono Q column (5 \times 50 mm), and nucleotides were separated by a linear gradient from 0% solvent B (300 mM NaCl in 10 mM BTP, pH 7.5)/100% solvent A (10 mM BTP, pH 7.5) to 100% solvent B in 30 min at a flow rate of 0.75 mL/min, monitored at 260 nm. To obtain radiolabeled myristoylated GCAP1-⁴³⁷⁻¹⁰⁵⁴GC1, the cells were grown in the presence of [9, 10(n)-³H]myristic acid (0.5 mCi/15 mL of cell culture).

Gel Filtration, SDS-PAGE, and Immunoblotting. The supernatant containing soluble recombinant GC (0.25 mL) was loaded on a Superose-6 gel filtration column (10 \times 300 mm, Pharmacia), equilibrated with 10 mM BTP, pH 7.5, containing 150 mM NaCl and 0.05% BSA at a flow rate of 0.5 mL/min. Fractions (0.5 mL) were collected, and aliquots of 40 μ L were tested for GC activity. The proteins were identified by SDS-PAGE using 15% polyacrylamide gels. For immunoblotting, membranes were blocked with 2% BSA in 20 mM Tris, pH 8.0, containing 150 mM NaCl and 0.05% Tween 20, and incubated for 1 h with primary antibody at a dilution of 1:1000. Cross-reacting anti-GC1 and -GC2 antibody was raised against the conserved dimerization domain (26), while GCAP1- and GCAP2-specific antibodies were generated as described previously (27).

Immunocytochemistry. Insect cells were immersed in chilled 4% formaldehyde in PBS for 2 h. Antibody labeling was assessed by indirect immunofluorescence. Fixed cells were incubated in blocking buffer (PBS containing 3% BSA and 0.2% Triton X-100, pH 7.0) for 30 min at room temperature to reduce nonspecific labeling, washed with PBS, incubated for 1.5 h with rabbit polyclonal antibody (UW28, anti-dimerization domain of GC1 at 1:100 dilution)(26), washed 3 times with PBS, incubated for 30 min with indocarbocyanine (Cy3)-conjugated goat anti-rabbit antibody (1:200 in PBS), extensively washed with PBS, mounted in 5% *n*-propyl gallate in glycerol, and examined under fluorescence microscopy at 568 nm.

RESULTS AND DISCUSSION

Studies of photoreceptor GC are hindered by several biochemical properties that are difficult to eliminate or control. In general, photoreceptor GCs are poorly extractable with detergents; they are expressed at low levels, sensitive to inactivation during biochemical manipulations, and in ROS, difficult to assay as a consequence of the opposing phosphodiesterase activity. GCs are stimulated by small Ca^{2+} -binding proteins, GCAPs, in a reaction that is highly sensitive to various factors, such as detergents or salts. In particular, the sensitivity to detergent does not allow for a clear determination of whether GCAP activation involves dimerization of GC, as documented to other sTMRs. Inability to reconstitute GC modulation with GCAPs using purified components could also indicate that other proteins, factors of structural elements of ROS, or heterologous expression cell systems are needed for this regulation. Therefore, our approach was to generate a soluble fusion protein that would circumvent these problems (Figure 1).

Active Truncated Forms of GCs and Constitutively Active Fusion Proteins between GCs and GCAPs. Recombinant GC1 is associated with the membranes and requires detergent for solubilization. The basal activity of recombinant GC1 expressed in insect cells was 0.02 ± 0.005 nmol/min, and was stimulated 10–20-fold by addition of purified GCAP1 and GCAP2 (Figure 2A) [reviewed also in ref (3)]. The intracellular fragment of GC1 (437–1054) was active, suggesting that the extracellular domain is nonessential for GCAP stimulation as was previously postulated (28, 29). Full-length GC1 and ⁴³⁷⁻¹⁰⁵⁴GC1 activities (IC_{50} equal to 225–230 nM) stimulated by soluble GCAP1 had comparable activity versus $[\text{Ca}^{2+}]$ relationships

(Figure 2A), and similar to the activation of ROS GCs by GCAP1(25). Further systematic truncation of the kinase-homology domain (KHD) led to a 468–1054 product that was stimulated by exogenous GCAP1, but not GCAP2 (data not shown). Additional truncation rendered the GC fragment inactive, indicating that the KHD either is needed for the proper folding of active GC1, is important for GCAP binding (Figure 1), or is required for GC activity, as suggested previously (28). In total, 51 different constructs were generated and tested in this study. All mutations at the C-terminus of GC, such as the addition of a linker (see below), GFP, His₆-tag, truncation of the last 6–10 amino acids, led to inactivation of the cyclases and were not useful for further analysis. Shortening the linker also led to inactive cyclase constructs. Therefore, mutants that provided additional information on the properties of GCs and GCAPs are only discussed in this report.

Based on these deletion experiments, fusion proteins were generated between selected CBPs and GCs (Figure 1). The fusion proteins were active when CBPs were added to the N-terminus of GC, while the addition of a 35 amino acid long linker (see Materials and Methods) to the GC1 and GC2 C-termini led to inactivation of the enzymes, without significant changes in the expression levels of the constructs (data not shown). Shortening of the GC construct by 44 amino acids from position 468 (Figure 1) and longer systematic deletions (data not shown), as observed for truncated GC1, led to cyclase inactivation.

Although GCAP1/2–GC1/2 constructs were constitutively active at 46 nM [Ca²⁺]_{free} with IC₅₀ = 220–255 nM [Ca²⁺] (Figure 2B,C), further addition of exogenous GCAP1 or GCAP2 increased the GC activity by 10–20%, suggesting that not all molecules in the GCAP–GC complexes are fully enzymatically productive and some are in a dissociated form with the regulatory site of GCs accessible for soluble free GCAPs. Moreover, at low [Ca²⁺], guanylyl cyclase-inhibitor protein (GCIP), a CBP related to GCAP, competes with GCAP1 in the GCAP1–^{437–1054}GC1 complex (Figure 2B). This observation confirms that the binding of GCAP1 to GC1 in the fusion protein is of low affinity, similar to stimulation in ROS membranes. Both binding sites for GCAP1 and GCIP, therefore, must overlap at least partially.

The inhibition of GC1 at high [Ca²⁺] required myristoylated GCAP1 (23), and when the recognition site for myristoyl-transferase was disabled by the addition of His₆-tag, enhanced cyclase activity was observed in high [Ca²⁺], which is typically reduced to basal levels by the myristoylated Ca²⁺-free form of GCAP1 (Figure 2B). These results indicate that the functional role of the N-terminal domain of GCAP1 in the inhibition of GC1 is independent of membrane anchoring.

Photoreceptor GC1 has been reported to be stimulated ~2-fold by 1–2 mM ATP, while higher concentrations of ATP decreased GC activity, most likely by competing with the substrate, GTP (30). To assess if the fusion protein still responds to ATP, increasing concentrations of this nucleotide were included in the assays. GC responsiveness to ATP was preserved both for the truncated ^{437–1054}GC1 and for the fusion GCAP1–^{437–1054}GC1. At 0.5–1.5 mM ATP, the GC constructs were activated and inhibited at higher concentrations (Figure 2B, inset). As a result, the truncated GC1 fully preserved the responsiveness to ATP, indicating that the membrane component is not needed for this regulation as well.

The GCAP1–^{437–1054}GC1 properties in response to an increase in [Ca²⁺] were also comparable to those of GCAP2–^{437–1054}GC1, GCAP1–^{437–1054}GC2, and GCAP2–^{437–1054}GC2 (Figure 2C). These results indicate compatibility between GC1 and GC2/GCAP1 and GCAP2, suggesting that underlying mechanisms of activation are similar for both GCAPs and GCs. This finding is in contrast to other observations in reconstituted systems using the mutated and native GCs and GCAPs [reviewed in ref (31)]. These differences between membrane-bound and free systems could result from the alteration in affinities between these two nonlinked

components and changes in effective concentrations in a milieu of other proteins on the surface of the membranes as compared to soluble proteins. As expected, not all CBPs have the stimulatory effect on GC1. When CaM was fused to $^{437-1054}$ GC1, only a basal activity was observed at all tested $[Ca^{2+}]$, and the cyclase activity was enhanced in low $[Ca^{2+}]$ upon addition of exogenous GCAP1 (Figure 2D). Together, these data show that the fusion proteins between GCs and GCAPs display all major enzymatic characteristics of the native system in ROS. Therefore, these constructs were further evaluated to understand the mechanism of cyclase modulation by GCAPs.

Soluble Forms of GC Fusion Proteins. The fraction of active and soluble $^{437-1054}$ GC1 was estimated by the enzymatic assays in membrane suspensions and soluble fractions, and fusion proteins were visualized by immunoblotting with anti-GC1 antibodies. A major fraction of all fusion proteins or truncated GCs was soluble, in contrast to full-length GC1 (Figure 3A). The production of the soluble active products in insect cells is different from the expression of insoluble truncated GC1 obtained in HEK293 cells (29). These differences suggest that the insolubility of the intracellular domain of GC1 may be due to either differences in the expression system (insect cells vs HEK293 cells) or differences in GC1 intracellular sequences (human vs bovine), rather than a general feature of this GC domain. It is worth noting that the intracellular domain of GC type C is also soluble in insect cells (32). The cellular localization of $^{437-1054}$ GC1 showed cytosolic distribution in contrast to plasma membrane association of GC1 (Figure 3B). To determine if $^{437-1054}$ GC1 is myristoylated, the fusion protein was produced in the presence of [3 H]-myristic acid. Because [3 H-myristoyl]- $^{437-1054}$ GC1 was eluted in fractions that corresponded to the GC-stimulated activity at low $[Ca^{2+}]$, and no radioactivity was detected in the fraction for the control mutant with the disabled myristylation site, these results and inhibition of GC1 at high $[Ca^{2+}]$ (Figure 2B) suggest that the fusion protein is myristoylated (Figure 3C).

The GCAP1- $^{437-1054}$ GC1 fusion proteins were subjected to various purification schemes. For example, His₆-GCAP1- $^{437-1054}$ GC1 was partially purified by Ni^{2+} metal-affinity chromatography (data not shown). Enzymatic properties (such as $[Ca^{2+}]$ sensitivity) of purified fractions of GCAP-GC fusion proteins were indistinguishable from regulation of membrane-associated GC in ROS or HEK293 membranes. Strep-GCAP1-GC1, containing the MASN-WSHPQFEK-GG-tag sequence, can be developed using the streptavidin affinity purification method (21). Another construct with an antibody recognition tag, PPE-GCAP1- $^{437-1054}$ GC1, containing the PRGPDRPEGIEE sequence, was purified to apparent homogeneity by immunoaffinity chromatography (Figure 3D). It appears, therefore, that no other membrane component is needed for GC1 stimulation by GCAP1 and that these proteins interact directly, rather than through adapter molecules such as cytoskeletal proteins, and that no membrane component is needed for the activation process.

The expression of fusion proteins has been extensively studied in *E. coli* without success. The vast majority of the expressed protein formed insoluble aggregates, and both the soluble fraction and the insoluble fraction, renatured from urea, were without GC activity. Further improvements, such as simplifying the purification procedures, are required for increasing protein levels in various heterologous expression systems, including insect cells, before structural studies could be embarked on.

Mechanism of GC Stimulation by GCAPs. To probe the mechanism of GC stimulation, we employed GCAP1- $^{437-1054}$ GC1 protein; however, other constructs yielded similar results. To further investigate the affinity of GCAP1 for GCAP1- $^{437-1054}$ GC1 in a mixture of free components and as a fusion protein, the GC activity was measured in the presence of increasing concentrations of the constitutively active GCAP1 mutant at high $[Ca^{2+}]$ (33) (Figure 4A). In these conditions, without the constitutively active mutant, native GCAP1 inhibits GC activity

(Figure 2A). For GCAP1 to GCAP1_{437–1054}GC1, the EC₅₀ for stimulation with the GCAP1 mutant was comparable (~0.8 μM), and similar in our conditions to the EC₅₀ for membrane-bound GC and GCAP1 (~1.5 μM) (33), supporting again the hypothesis that GCAP1 in GCAP1_{437–1054}GC1 forms a freely dissociable complex and that GCAP1 in the fusion protein can be out-competed by soluble GCAPs. Although the activation of GCAP1_{437–1054}GC1 could be intermolecular (Figure 2B,D), in diluted conditions, most of the activation occurs through an intramolecular mechanism (Figure 4B) because dilution of GCAP1_{437–1054}GC1 had only a minor effect on the activity as compared to strong inhibition by the dilution of GCs in ROS in the presence of exogenous GCAP1.

GCAP1_{437–1054}GC1 formed a homodimer of intracellular domains as judged by the gel filtration method in both low and high [Ca²⁺] (Figure 4C). These results are consistent with the observations made using other methods for photoreceptor GCs in membranes (14, 34, 35) and other membrane-associated GCs (12, 32). X-ray structure determination of the soluble extracellular domain also suggests a dimeric form of this part of GC (36), and that the extracellular and intracellular domains permit a set of interactions that brings the two subunits together. The regulatory interactions of GCAPs with the effector GC molecules cause conformational changes that produce the most efficient enhancement in the catalysis, rather than physical dimerization. Recently, we postulated an activation model that assumes changes within the flexible central helix of GCAPs upon Ca²⁺ dissociation, causing relative reorientation of two structural domains containing a pair of EF-hand motifs, and thus switching its partner, GC, from an inactive to an active conformation (37). The essential components required to transform an inhibitory to an activating protein are contained within the N-terminal region of GCAP1 (residues 1–43) (23, 38, 39). These results deviate from the mechanism proposed for the photoreceptor GC regulation, in which GCAP2 promotes a dimerization of GCs in the Ca²⁺-free form (35).

In summary, active and soluble GCAP–GC fusion proteins were generated and characterized in biochemical assays. The interaction of GCs with GCAPs did not require any membrane components or adapter proteins. The soluble GCAP–GC fusion proteins are dimers in all tested conditions, and the GC activity showed typical Ca²⁺-dependent stimulation that was further enhanced by ATP. The myristoyl group of GCAP1 appeared to be critical for the inhibition of GCs at high [Ca²⁺]. Expression of the active soluble intracellular GC domain in a complex with GCAPs opens new possibilities for using these constructs in structural studies. Thus, the sensitivity of GCs and GCAPs to various reagents, such as salt and detergents, and the relatively low affinities compared to CaM and its target proteins could be overcome by fusing these two components. This raises the possibility to determine the high-resolution structures of the fusion proteins in both Ca²⁺-free and Ca²⁺-bound forms. Their critical role in the physiology of photoreceptors and vision makes GC–GCAP systems an attractive target for further studies. These studies may also influence further progress on other sTMRs and their interactions with modulatory proteins.

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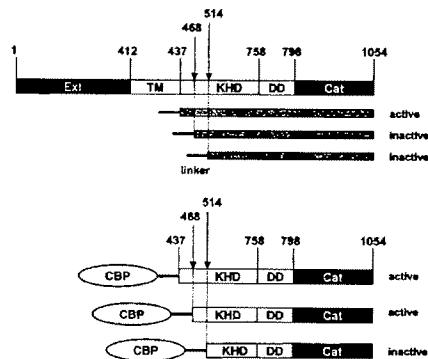
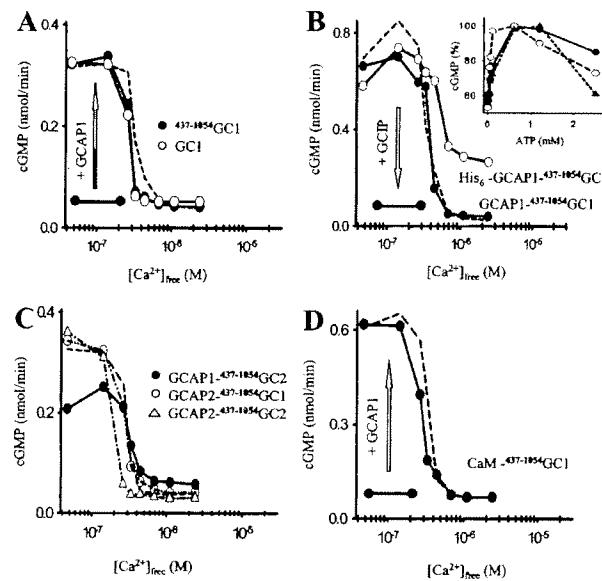
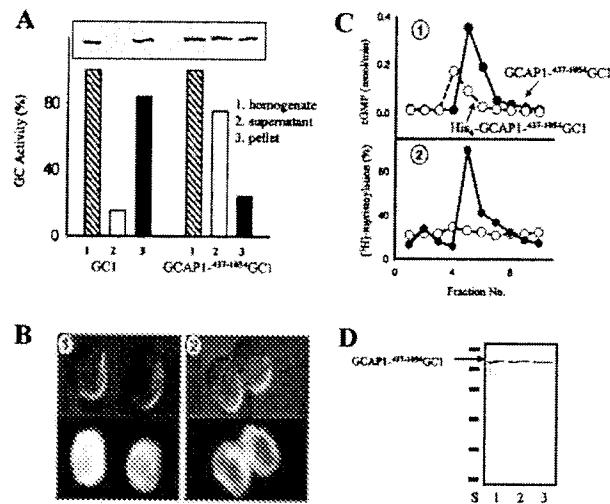


Figure 1.

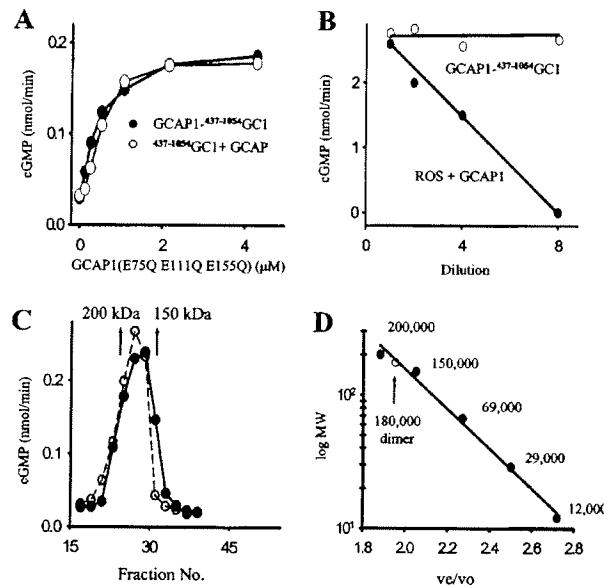
Schematic representation of truncated GCs and CBP–GC fusion proteins. The scheme represents Ext (extracellular), TM (transmembrane), KHD (kinase-homology), DD (dimerization), and Cat (catalytic) domains of GCs. The GC activity was assayed in the whole cell homogenate and in the detergent-free extracts in the presence of GCAP1 at 46 nM [Ca²⁺]_{free}.

**Figure 2.**

Activity of GC constructs as a function of $[Ca^{2+}]_{free}$. (A) Activity of GC for full-length GC1 and truncated $^{437-1054}GC1$ (enzyme was stimulated by addition of exogenous, recombinant GCAP1). IC_{50} for GC1 was 230 ± 40 and 225 ± 34 nM for $^{437-1054}GC1$ ($n = 3$). (B) Activity of GC for GCAP1- $^{437-1054}GC1$ and His₆-GCAP1- $^{437-1054}GC1$ (arrow shows competition of GCIP with GCAP1 in GCAP1- $^{437-1054}GC1$ fusion protein at low $[Ca^{2+}]_{free}$). IC_{50} for His₆-GCAP1- $^{437-1054}GC1$ was 220 ± 34 nM. Inset: ATP titration of GC constructs (solid circles, GCAP1- $^{437-1054}GC1$; open circles, $^{437-1054}GC1 + GCAP1$; open triangles, GC1 + GCAP1) in low $[Ca^{2+}]_{free}$. Similar results were obtained in three independent measurements. (C) Activity of GC for GCAP1- $^{437-1054}GC2$, GCAP2- $^{437-1054}GC1$, and GCAP2- $^{437-1054}GC2$. IC_{50} values for GCAP2- $^{437-1054}GC1$ and GCAP2- $^{437-1054}GC1$ were 245 ± 74 and 255 ± 30 nM, respectively. (D) Activity of GC for CaM- $^{437-1054}GC1$ after stimulation by addition of GCAP1. Arrow shows recovery of GC1 activity by reconstitution with GCAP1 in low $[Ca^{2+}]_{free}$. IC_{50} for CaM- $^{437-1054}GC1$ was 195 ± 15 nM. The dashed line represents stimulation of ROS GCs by GCAP1. In all panels, the dashed line represents stimulation of ROS GCs by GCIP.

**Figure 3.**

Properties of GCAP1-GC1 fusion proteins in cell culture, biochemical assays, and protein purification. (A) Solubility of GC1 constructs. GC was prepared as described under Materials and Methods. Activity (as a percent of maximal activity) was measured in membrane and soluble cell fractions. Similar results were obtained in four independent measurements. Inset: Immunoblot analysis of GC1 solubility using polyclonal anti-GC1 antibodies. (B) Localization of GCAP1-437-1054-GC1 fusion protein in cytoplasm (1) and full-length GC1 in the membrane fraction (2) (upper panels corresponded to image in bright light, bottom panels show fluorescent image). (C) Gel filtration chromatography of GCAP1-437-1054-GC1 and His₆-GCAP1-437-1054-GC1 (Materials and Methods) and correlation with GC stimulation activity. Panel 1, GC activity profile; panel 2, incorporation of the myristoyl group into GCAP1-GC1 mutants. (D) Purification of PPE-GCAP1-437-1054-GC1. The GC1 fusion protein was purified as described under Materials and Methods. The purity was assessed by SDS-PAGE (lane 1) and by immunoblotting using anti-GC1 antibody (lane 2) and GCAP1 antibody (lane 3). The standards are (in kDa): phosphorylase B, 116; BSA, 80; ovalbumin, 52.5; carbonic anhydrase, 34.9; soybean trypsin inhibitor, 29.9; lysosyme, 21.8.

**Figure 4.**

Intramolecular and dimeric nature of GCAP1-GC1-stimulated activity at low $[Ca^{2+}]_{free}$. (A) Competition of GCAP1- $^{437-1054}GC1$ and $^{437-1054}GC1$ with GCAP1(E75Q E111Q E155Q) at 2 μM $[Ca^{2+}]_{free}$. The EC₅₀ values were 0.8 ± 0.1 and $0.85 \pm 0.1 \mu M$ for GCAP1- $^{437-1054}GC1$ and $^{437-1054}GC1$, respectively. (B) Dilution effect on GCAP1- $^{437-1054}GC1$ and GCAP1/ROS GC1 activities. Samples were diluted 0, 2, 4, and 8 times with increasing concentrations of all reagents in the assay at constant amounts of protein. Results are an average of two measurements. (C) Gel filtration chromatography. GCAP1- $^{437-1054}GC1$ was loaded at 5 μM $[Ca^{2+}]_{free}$ or with (gray circles and dashed line) addition of 1 mM EDTA (solid circles) on a Superose-6 column as described under Materials and Methods. Arrows show standards: α -amylase (200 kDa) and alcohol dehydrogenase (150 kDa). (D) The calibration curve for the gel filtration column. The standard proteins and compounds used were NaN₃, cytochrome *c* (12 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (67 kDa), alcohol dehydrogenase (150 kDa), α -amylase (200 kDa), and blue dextran (void volume, 2000 kDa) as the high molecular mass standard. The protein standards were detected at 280 nm, blue dextran at 450 nm, and azide at 260 nm. ve/vo is a ratio of the elution volume to void volume. The GC fusion protein eluted at a volume that corresponded to 180 kDa. Similar relationships for the retention time and molecular mass were obtained in three independent experiments.



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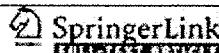
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Ultracytochemistry as a tool for the study of the cellular and subcellular localization of membrane-bound guanylate cyclase (GC) activity. Applicability to both receptor-activated and receptor-independent GC activity.

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Membrane-bound guanylate cyclase activity was detected by ultracytochemistry at the electron microscope level in several mammalian tissues. The technique used in these studies allows the detection of active enzyme at the membrane site where it is located. In a few cases, such as normal and regenerating peripheral nerves and placenta, membrane-bound guanylate cyclase could be detected in the absence of stimulators of enzyme activity. However, in the majority of these studies membrane-bound guanylate cyclase was investigated following stimulation with natriuretic peptides, guanylin, or the Ca^{2+} sensor proteins, S100B and S100A1. In general, membrane-bound guanylate cyclase was localized to plasma membranes, in accordance with the functional role of this enzyme. Yet, in secretory cells the enzyme activity was localized on intracellular membranes, suggesting a role of membrane-bound guanylate cyclase in secretory processes. Finally, S100B and S100A1 were found to colocalize with membrane-bound guanylate cyclase on photoreceptor disc membranes and to stimulate enzyme activity at these sites in dark-adapted retinas in a Ca^{2+} -dependent manner. The results of these analyses are discussed in relation to the proposed functional role(s) of this enzyme.

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